Regulation of cytochrome c oxidase subunit 1 (COX1) expression in Cryptococcus neoformans by temperature and host environment

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In the study of differential gene expression of Cryptococcus neoformans, a transcript of COX1 (cytochrome oxidase c subunit 1) was identified in a serotype A strain. The transcript was upregulated at 37 °C compared to 30 °C and expressed by yeasts infecting the central nervous system. Northern analysis of COX1 from the serotype A strain revealed two polycistronic transcripts, a temperature-upregulated 2.3 kb transcript and a 1.9 kb transcript that was not affected by temperature. In contrast, COX1 in a serotype D strain showed only a 1.9 kb polycistronic transcript plus a 1.6 kb monocistronic message, and temperature had no effect on the transcripts. The sequence of COX1 revealed similar coding regions between the two strains, but the serotype D strain had five introns whereas no introns were found in the serotype A strain. The serotype D strain had reduced growth rates compared to the serotype A strain at 37 °C, but in an AD hybrid strain the serotype D COX1 gene could support efficient high temperature growth. These studies have revealed mitochondrial molecular differences between serotype A and D strains which show evolutionary divergence. It will be important to determine whether differences in mitochondrial structure and function can influence cryptococcosis.

INTRODUCTION

Cryptococcus neoformans is an encapsulated yeast with a propensity for invading the central nervous system of healthy and immunocompromised individuals. Since the beginning of the AIDS pandemic (in the early 1980s), the occurrence of cryptococcal infection has increased sharply, and although several antifungal drugs, including amphotericin B, fluconazole, fluconazole and itraconazole, have shown moderate efficacy in treatment of this infection, a significant number of relapses and treatment failures still occur (Perfect, 1989). The discovery of new antifungal agents which selectively kill or inhibit the growth of C. neoformans in the host is needed for more effective management of this serious and sometimes fatal infection.

Recent studies into the pathobiology of C. neoformans have established effective molecular tools to examine the pathogenicity of this fungus and thus identify potential genetic targets for drugs (Chang et al., 1996; Chang & Kwon-Chung, 1994; Lodge et al., 1994; Odom et al., 1997; Salas et al., 1996). Several C. neoformans genes disrupted by targeted gene replacement have now been shown to be associated with the pathogenicity of this yeast. Some examples are those for capsule formation (Chang et al., 1996), laccase for melanin production (Salas et al., 1996), urease (Cox et al., 2000), phospholipase (Cox et al., 2001) and mannose metabolism (Wills et al., 2001). Signal transduction pathways for control of virulence include calcineurin (Odom et al., 1997), GPA1 (Alspaugh et al., 1997), PKA1 (D’Souza et al., 2001) and RAS1 (Alspaugh et al., 2000), which regulate genes for the three major virulence phenotypes of capsule, melanin and growth at 37 °C.

The specific hypothesis for this study is that detectable changes in gene expression directed by certain pathobiological conditions may identify genetic loci that are important for the virulence components of C. neoformans (Liang & Pardee, 1992; Mahan et al., 1993; Schena et al., 1995; Velculescu et al., 1995; Wodicka et al., 1997). We used differential display RT-PCR technology (Liang & Pardee, 1992) to identify C. neoformans transcripts that were upregulated by exposure to 37 °C as compared to 30 °C. Furthermore, transcripts were captured and identified from yeast cells taken directly from the cerebrospinal fluid of immunosuppressed rabbits during meningitis. In this screen, one partial transcript (p1.1dd), which satisfied both criteria of increased expression in response to elevated...
temperature (37 °C) and expression in a mammalian host, shared extensive homology with the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene. COX1 encodes an important enzyme involved in the oxidation phosphorylation pathway and thus energy production for C. neoformans. Implications concerning the upregulation of this mitochondrial polycistronic transcript during the stress of certain temperatures and its evolution in serotype A and D strains are revealed.

METHODS

Yeast strains and media. C. neoformans strains used in these studies were H99, a serotype A MATa (C. neoformans var. grubii) clinical isolate that has been shown to produce chronic meningitis in corticosteroid-treated rabbits (Perfet et al., 1980), and JEC21, a serotype D MATa (C. neoformans var. neoformans) strain derived from a cross between 3501 (MATa) and 3502 (MATa) as described previously (Kwon-Chung et al., 1992). For genetic crosses to generate AD hybrid strains, AAC51 (MATa, ura-5) derived from H99 and JEC30 (MATa, lys-) derived from JEC20, a serotype D MATa and congeneric to JEC21, were used. Serotype A isolates (ATCC 34869; CDC-B4962, Zaire; CDC-Y195, Brazil; CDC-Y288, Canada) and serotype D isolates (ATCC 34875; ATCC 24067; CDC-Y286, Canada; CAP 67–2) were used to compare COXI intrinsic structures within strains of each serotype. These isolates were obtained from Mary Brandt, Centers for Disease Control and Prevention, USA, and have been described previously (Brandt et al., 1993; Jacobson et al., 1982). The medium used to propagate yeast cells was 1 % yeast extract, 1 % peptone and 2 % glucose (YPG). Solid medium containing 2 % agar. Yeast cells were maintained on YPG agar plates at 1982). The medium used to propagate yeast cells was 1 % yeast AD hybrid strains, AAC51 (MATa, ura-5) derived from JEC20, a serotype D MATa and congeneric to JEC21, were used. Serotype A isolates (ATCC 34869; CDC-B4962, Zaire; CDC-Y195, Brazil; CDC-Y288, Canada) and serotype D isolates (ATCC 34875; ATCC 24067; CDC-Y286, Canada; CAP 67–2) were used to compare COXI intrinsic structures within strains of each serotype. These isolates were obtained from Mary Brandt, Centers for Disease Control and Prevention, USA, and have been described previously (Brandt et al., 1993; Jacobson et al., 1982). The medium used to propagate yeast cells was 1 % yeast extract, 1 % peptone and 2 % glucose (YPG). Solid medium contained 2 % agar. Yeast cells were maintained on YPG agar plates at 4 °C during studies.

Isolation of differentially upregulated RNA. To identify genes upregulated by mammalian host temperature, H99 cells were grown to mid- to late-exponential phase in YPG broth at 30 and 37 °C, pelleted and washed three times in cold 500 mM NaCl/50 mM EDTA. For in vivo regulated gene expression, H99 cells were propagated on YPG agar plates for 72 h at 30 °C, collected on cotton swabs and suspended in PBS (pH 7–4) at a cell density of 4 × 10^7 ml⁻¹. Approximately 2 × 10^8 viable yeast cells were inoculated intracerebrally into each of seven New Zealand White male rabbits that had received an intramuscular injection of 5 mg hydrocortisone acetate kg⁻¹ (25 mg ml⁻¹; Schein Pharmaceuticals) 24 h earlier and then daily during the infection. Yeast cells were recovered from the cerebrospinal fluid at days 2 and 7 of the infection, placed on ice and then washed two times in cold water in an attempt to eliminate host cells. Both in vitro (10^8) and in vivo (~5 × 10^7, comprising a separate pool of day 2 and day 7) grown cells were stored at −70 °C in a pellet. RNA was isolated using the Fast RNA Red kit (Bio 101) along with glass bead agitation for 20 s at a speed rating of 6 in a Savant Fast Prep 120 Instrument. The tubes were then chilled on ice for 40 s. This procedure was repeated three times. To remove contaminating polysaccharide, the RNA was precipitated with LiCl (Fast RNA Red kit), pelleted, washed once in 70 % diethyl pyrocarbonate (DEPC)-treated ethanol, dissolved in 50–100 µl of DEPC-treated water at a concentration of ≥1 µg µl⁻¹ and stored at −70 °C. Due to the low yield of yeast cells recovered from the cerebrospinal fluid, RNA was only quantified from in vitro grown cells by absorbance at 260 nm. Usually, 150–200 µg of total cellular RNA was recovered from 10^9 yeast cells grown in vitro. mRNA was extracted from in vitro total RNA with the use of the Oligotex mRNA minikit (Qiagen). RNA was also isolated from JEC21 cells grown at 30 and 37 °C using the Fast RNA Red kit.

Differential display RT-PCR. Each H99 RNA sample was treated with DNase I (amplification grade; Gibco-BRL) and then transcribed by reverse transcriptase using the RNA Image kit (GenHunter) to first strand cDNA using three different one-base anchored oligo[dT] primers (dT11-A, dT11-C, dT11-G; 200 ng of in vitro RNA and one-third of the pooled day 2 or day 7 in vivo RNA per anchored primer). First strand cDNAs were then amplified by PCR with the specific anchored primer and a 13-mer arbitrary primer (API) from the kit in the presence of [32P]dATP (New England Nuclear). The PCR conditions were 94 °C for 30 s, 40 °C for 2 min and 72 °C for 30 s (40 cycles), followed by elongation at 72 °C for 5 min. The cDNAs were then distributed on a 6 % denaturing sequence gel for analysis. High temperature (37 °C) upregulated cDNAs were compared to 30 °C and cDNAs present in in vivo cells. The cDNAs with increased expression at elevated temperature and detected from in vivo cells were eluted from the gel and reamplified as described by the RNA Image protocol. The cDNAs were then blunt-ended with T4 DNA polymerase and cloned into the EcoRV site in pBluescript KS±.

Molecular biology techniques. Isolation of DNA, representing a mixture of nuclear and mitochondrial DNA, from yeast sphe- roplasts was performed according to Toffaletti et al. (1993). An EMBL3 genomic library of C. neoformans H99, which also carries mitochondrial genes, has been described previously (Cox et al., 1995). Plaque lifts were performed according to Sambrook et al. (1989). DNA sequencing methodology for cDNA clones was by dideoxy chain termination with a Sequenase version 2.0 kit (US Biochemical).

For Northern analysis, total cellular RNA (15 µg lane⁻¹) and mRNA (1 µg lane⁻¹) were electrophoresed on a 1.5 % formaldehyde/agarose gel. RNA was blotted onto Nytran membranes (Schleicher & Schull) and hybridized to 32P-labelled DNA probes according to Sambrook et al. (1989). Quantitative assessment of the RNA transcripts was carried out in a phosphor imager and the bands were analysed using IMAGE QUANT software (Molecular Dynamics).

Karyotype analysis was performed according to Perfet et al. (1989) and electrophoresed at 15 °C under the following conditions. For nuclear chromosome analysis: 125 V for 20 h (ramped from 50 to 130 s) followed by 125 V for 41 h (ramped from 170 to 300 s). For mitochondrial DNA analysis: 125 V for 20 h (ramped from 50 to 130 s). The chromosomes were stained with ethidium bromide and then blotted onto Nytran membranes as described by Sambrook et al. (1989).

DNA probes were made with the Random Primer Labelling kit (Gibco-BRL) and [32P]dCTP (New England Nuclear) using, as templates, the COXI p1.1dd cDNA (420 bp), various portions of the ATP9–COXI–ATP8 transcriptional unit (300–500 bp) and a fragment of the actin gene (Stxl-restricted 450 bp).

Isolation and sequencing of the C. neoformans COXI gene encoding cytochrome c oxidase subunit 1. A genomic library of H99 in EMBL3 was screened with the 32P-labelled 420 bp cDNA fragment p1.1dd containing COXI. Positive clones were passed through three to four repeated screenings to obtain single clones. Several of these clones were amplified using the plate lysate procedure according to Sambrook et al. (1989) and stored at 4 °C. Genomic clones C-2 and C-4 were digested with SalI and EcoRI, respectively, producing single hybridizing fragments of 6.0 kb (C-2) and 3.0 kb (C-4). These fragments were then subcloned into pBluescript KS± for sequencing (Oefner et al., 1996). Partial sequences of C-2 and C-4 were obtained using primers from the corresponding cDNA upregulated transcript. Primers 1Rda
Expression of COX1 in C. neoforms

(5'-CGG ATC TCA TCT TCA TCC-3') and #2Rda (5'-CAC CAA TGA ATA GAG TCC AG-3') were used to sequence genomic clones C-2 and C-4, respectively. Additional primers were used to sequence the complete C-2 and C-4 genomic clones. These primers were Da1 (5'-GCA CCC ATT GAA AGT ACG TAG TGG-3'), Da3 (5'-GCA ATA TAT ACC ATC COG-3'), Da5 (5'-CCA GGA GCT CGC ATG TTA A-3'), Da7 (5'-CGG TGA GAT GTA GCG ATT AC-3'), Da9 (5'-CGG TTC CTC TCA GGG GAT TA-3') and Da11 (5'-GGG GAG TAT AGG TAT TG-3') for clone C-4, and Da4 (5'-CCA CTA GCT ACT TCT AAT GGG TGC-3'), Da6 (5'-CCA GTT GCA TCT ATG AAC CC-3'), Da8 (5'-GTC ATG ATA CGT ACT ACC TT-3'), Da10 (5'-CGA AAC AGT CAA TAT AGG TT-3') and Da12 (5'-CAC TGC AGC ACT ATC ATA-3') for clone C-2. The complete mitochondrial sequence of strain H99 was obtained from a whole shotgun library prepared in pUC18 using 1996). DNA was prepared using Rev Prep (Gene Machines) and sequenced to more than 10-fold coverage of the mitochondrial genome using an ABI model 3700 automated DNA sequencer (Applied Biosystems).

In vitro growth kinetics of C. neoforms at high temperatures. An overnight YPG broth culture of each strain of C. neoforms [serotype A (H99) and serotype D (JEC21)] yeast cells was inoculated into YPG broth at a final cell density of 10^6 ml^-1. The cells were allowed to incubate with shaking at 37 or 39°C for 48 h. At various times after inoculation (0, 6, 12, 24, 36 and 48 h), samples were taken from the broth and diluted on YPG agar plates for quantitative assessment of yeast counts. All cultures were performed in duplicate. Generation times were determined during exponential phase growth (37°C, 12–24 h; 39°C, 6–12 h).

Mating assay. Strains AAGC1 (MATa, ets-) and JEC30 (MATa, lys-) were pre-grown on YPG for 2–3 days. The two yeast strains were then mixed on V8 medium containing 5% (v/v) V8 juice, 0.05% KH2PO4 and 4% agar (pH 7.0), and incubated at 25°C in the dark for 7 days. Cells that produced hyphae were resuspended in sterile water, plated onto minimal medium agar without amino acids (Difco) with 2% glucose to select for prototrophic (URAS, LYS) hybrids and incubated at 30°C. Prototrophic colonies were then transferred to YPG plates at 39°C to identify colonies which grew well at this temperature.

RESULTS

Selection and sequence analysis of H99 serotype A transcripts upregulated by high temperature (in vitro) and the host’s environment (in vivo)

Differential display RT-PCR on strain H99 was used to identify differentially regulated transcripts induced at the physiological temperature of 37°C compared to 30°C under otherwise identical conditions. These transcripts were then compared by differential display RT-PCR to transcripts found in yeast cells from the subarachnoid space of infected rabbits at days 2 and 7 after the onset of meningitis (in vivo). We selected several of the upregulated (≥2×) cDNAs at 37°C compared to 30°C, which were also present in the in vivo exposed yeast cells at days 2 and 7 of the infection in the cerebrospinal fluid, to elute from the gel, reamplify and clone into pBluescript KS+ for further sequence analysis. Three cloned upregulated transcripts were sequenced and compared to genes present in GenBank. Two of these transcripts lacked homology with any known gene; however, one transcript, p1.1dd, showed >50% homology with the 3’ end of the cytochrome c oxidase subunit 1 (COX1) gene in Prototheca wickerhammii. Furthermore, this COX1 transcript was also found to be expressed in yeast cells recovered from the cerebrospinal fluid at days 2 and 7 of the infection by RT-PCR (Rude et al., 2002).

Sequence analysis of H99 positive genomic clones

Sequence analysis of two positive genomic clones (C-2 and C-4), which hybridized to the upregulated cDNA fragment of COX1 (p1.1dd), revealed a multi-gene arrangement from 5’ to 3’ that included the ATP6, ATP9, COX1, ATP8 and NADH2 genes, respectively, on a 3.8 kb fragment of DNA. All this sequence was re-confirmed by shotgun sequencing and annotation of the entire H99 mitochondrial genome. The COX1-encoding region consisted of 1584 bp showing 66% homology with the COX1 gene derived from Kluyveromyces lactis. Interestingly, the COX1 sequence of H99 revealed no apparent consensus intronic structures. Open reading frames (ORFs) containing genes for ATP9, ATP8 and NADH2 were considerably smaller, comprising 216, 144 and 477 bp, respectively, and were structured without introns. A partial sequence of the ATP6 gene revealed a 5’-truncated coding region of 453 bp. Further analysis of each ORF in various databases showed significant similarities with the homologous mitochondrial genes present in several organisms (Table 1).

This 3.8 kb region of mitochondrial DNA carrying the COX1 gene was then compared to mitochondrial DNA from a serotype D strain, JEC21, which was sequenced, annotated and deposited in the Stanford University database (http://www.stanford.edu; permission from Richard Hyman). Although there was a synteny of genes on the mitochondrial chromosome between the serotype A and serotype D strains, the COX1 gene was considerably larger in serotype D, spanning a region of approximately 6000 bp, and had five introns (Fig. 1).

To determine if the intronic structure was strain- or variety-specific, five unique strains of serotype A and D were assessed for COX1 introns. Primers, carrying exonic

<table>
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<tr>
<th>Organism</th>
<th>Identity to C. neoforms H99 (%)*</th>
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<tr>
<td>Saccharomyces cerevisiae</td>
<td>ATP9  60 COX1  64 ATP8  37 NADH2</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>ATP9  60 COX1  65 ATP8  45 NADH2</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>ATP9  64 COX1  66 ATP8  43 NADH2</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>ATP9  60 COX1  33 ATP8  37 NADH2</td>
</tr>
<tr>
<td>Prototheca wickerhammii</td>
<td>ATP9  66 COX1  43 ATP8  27 NADH2</td>
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* Amino acid translation of complete nucleotide sequence.
sequences homologous to both H99 (serotype A) and JEC21 (serotype D), were used that could PCR amplify the five introns of COX1. For serotype A strains, four of five strains had no introns. One serotype A strain (CDC-Y288) contained introns 1 and 2. For serotype D strains, three of five strains had five introns but two strains (ATCC 24067 and CDC-Y286) were missing intron 3 (data not shown).

Karyotype analysis

Although yeast mitochondrial mRNAs have been reported to lack poly(A) tails (Groot et al., 1974), these transcripts are extensively AU-rich and could easily anneal to an oligo[dT] primer and thus be reverse transcribed into first strand cDNA. Identification of the amplified p1.1dd cDNA fragment and COX1 as a mitochondrial gene was further confirmed by karyotype analysis. Briefly, chromosome preparations from H99 yeast cells were electrophoresed at two different time intervals producing distinctive mitochondrial or nuclear chromosomal bands. A karyotype blot was then probed with a partial sequence of COX1. Sequences hybridizing to this probe were present on both the smaller band of 25 kb, which represents the known size of the H99 mitochondrial chromosome (Fig. 2a), and a 1500 kb chromosome (Fig. 2a, b). As expected, the actin probe hybridized to sequences present on its known location, on a 2200 kb chromosome, in H99 (Fig. 2a, b). Karyotype blots were also probed with DNA fragments carrying sequences of the ATP9 or ATP8 genes which flank COX1 at the 5′ or 3′ ends, respectively. Hybridization patterns generated with each probe were identical to the pattern observed with the COX1 probe, with positive signals on both mitochondrial and nuclear (1500 kb) chromosomes. In our twofold sequence coverage of the serotype A genome of C. neoformans, we found two partial sequences of 360 and 80 bp of COX1 in nuclear DNA. Likewise, a search of the C. neoformans serotype D sequence assembled at the Stanford Genome Technology Center (http://www-sequence.stanford.edu/) revealed two different segments of the COX1 mitochondrial gene integrated into the nuclear genome. One segment encodes 67 bp of the amino terminal end of the COX1 gene; the other segment encodes 11 bp from the middle of COX1. There was no evidence that these sequences represented an entire gene but they could have hybridized to the COX1 probe.

Northern analysis

Overexpression of the C. neoformans H99 COX1 gene at 37 versus 30˚C was confirmed by Northern blot analysis and quantified by use of a phosphor imager. A Northern blot of total RNA isolated from H99 cells grown at 30 and 37˚C was probed with either COX1 or a quantitative control gene of actin. As depicted in Fig. 3, the COX1 cDNA probe
hybridized to both a 1.9 kb and a 2.3 kb transcript; however, the signal intensity of the 2.3 kb transcript was three- to fourfold higher at 37 °C than at 30 °C. There was no significant difference in the signal intensity of the 1.9 kb transcript at 30 or 37 °C. Although the COX1-encoding region contains only 1584 bp, no transcript of this predicted size was detected by the COX1 probe.

In the yeast Saccharomyces cerevisiae, mitochondrial genes are polytranscribed into large precursor RNAs, which are subsequently processed into smaller intermediate and monocistronic transcripts (Costanzo & Fox, 1990; Grivell, 1989). To determine if the larger COX1 transcript in C. neoformans H99 is also polycistronic and encodes genes that cluster around COX1, Northern blots were also probed with the adjacent upstream (ATP9) or downstream (ATP8) genes. The predicted size of polycistronic transcripts carrying sequences encoding the ATP9, COX1 and ATP8 genes or the COX1 and ATP8 genes would be 2.3 and 1.9 kb, respectively. As revealed by Northern analysis, the ATP9 and ATP8 probes both hybridized to the 2.3 kb transcript, whereas the 1.9 kb transcript hybridized only to the ATP8 probe (data not shown). This hybridization pattern verifies the existence of a polycistronic transcript encoded by ATP9, COX1 and ATP8, which is either upregulated or less efficiently spliced by higher temperature. This polycistronic message is then processed to a dicistronic mRNA, containing the COX1 and ATP8 transcripts.

To ascertain if high temperature also increases the presence of this polycistronic (ATP9, COX1 and ATP8) transcript in a serotype D strain, Northern blots of total RNA isolated from JEC21 cells grown at 30 and 37 °C were probed with a fragment of the COX1-encoding region. Two transcripts of 1.9 and 1.6 kb in size were hybridized with equal intensity to the COX1 probe (Fig. 4, lanes 3 and 4). However, unlike H99, a 1.6 kb transcript, representing the predicted size of the mature COX1 mRNA, was also observed. Further analysis of Northern blots probed with the ATP9 and ATP8 genes showed the 1.9 kb COX1 transcript to be dicistronic, containing the ATP8 mRNA (data not shown).

To verify that mitochondrial transcripts encoded by COX1 are not polyadenylated, in vitro total RNA isolated from H99 cells was processed using oligo[dT]-coated latex beads and the eluted mRNA was probed with either the COX1 cDNA or the actin fragment. Although Northern analysis of the mRNA showed the presence of a 1.35 kb actin message, the COX1 mRNA was eliminated (Fig. 3), which supports the lack of polyadenylation of these mitochondrial mRNAs.

Growth rates of serotype A and D strains at 37 and 39 °C

Since the ability to grow and survive at high temperature is a major virulence factor for C. neoformans and mitochondrial polycistronic messages are differentially affected by temperature in serotype A (H99) versus serotype D (JEC21) strains, we assessed whether the growth rates for C. neoformans strains H99 and JEC21 were impacted by temperature. In vitro growth studies were performed at 37 and 39 °C. As shown in Fig. 5(a), H99 cells had a faster growth rate at 37 °C when compared to JEC21 cells cultured at the same temperature (generation times, 1.8 vs 2.5 h). When the temperature of the culture was increased to 39 °C,
the growth rate of H99 cells dropped approximately 2.5-fold (generation time, 4–6 h) whereas the JEC21 cells lost viability (Fig. 5b).

**AD hybrid strains with serotype D mitochondria**

With the use of the known uniparental transmission of mitochondria in *C. neoformans* (Xu et al., 2000a), AAC51 (MATa, *ura-5*) from H99 was crossed with JEC30 (MATa, *lys-*) from JEC20. During conjugation, AD hybrids were selected and colonies checked for presence of both serotype A and D alleles of GPA1, CLA1, STE12x and STE20a by PCR analysis (Lengeler et al., 2000). Colonies that were prototrophic and contained a mix of both genes were then checked by PCR for the presence of intronic structures in COX1. All hybrid colonies contained only the serotype D COX1 gene by PCR analysis. Several of these hybrid colonies had growth rates measured at 37 and 39 °C and were found to be the same as the wild-type H99 strain with the serotype A mitochondria (data not shown).

**DISCUSSION**

A dynamic change in an environmental cue (temperature) and the host’s environment (subarachnoid space of infected rabbits) provided signals for identifying differentially expressed genes which may play an important part in the growth of *C. neoformans* within the host. We have used differential display RT-PCR previously to identify an upregulated gene for isocitrate lyase whose product is the main controlling protein in the glyoxylate pathway. This gene was induced in the subarachnoid space during cryptococcal meningitis but the null-mutant did not have an attenuated virulence phenotype (Rude et al., 2002). This method is one of a series of techniques such as cDNA library subtraction (Duguid et al., 1988), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), *in vivo* expression technology (IVET) (Mahan et al., 1993) and DNA microarray (Schena et al., 1995) that have been developed to analyse gene expression. The advantage of these exploratory methods for the study of whole-genome expression is that there are no preconceived notions to focus genetic importance except for the specific environmental conditions. By the use of differential display RT-PCR, we observed differences in serotype A *C. neoformans* gene expression induced by changing environmental temperatures. From this we then focused on one transcript, COX1, which was upregulated by the mammalian host temperature (37 °C) and also expressed by the yeast *in vivo* throughout two weeks of cryptococcal meningitis. COX1 is a mitochondrial gene that encodes the cytochrome c oxidase subunit 1, a crucial enzyme involved in oxidative phosphorylation and thus energy production. It was hypothesized that this mitochondrial gene overexpression might be linked to an increased energy production and critically important to the survival of *C. neoformans* in a hostile host environment.

Identification of COX1 was substantiated by a partial sequence analysis of a positive genomic clone, showing extensive homology (>60%) with the 3′ end of COX1 in two other fungi, *Aspergillus nidulans* and *Neurospora crassa*, and eventual comparison of the entire sequence. Karyotyping and hybridization of chromosomes also confirmed the mitochondrial origin of COX1. However, COX1 and its adjacent upstream (*ATP9*) and downstream (*ATP8*) genes were also identified on a nuclear chromosome by hybridization. These data concur with several other studies showing that nuclear DNA of other eukaryotic organisms also contains sequences homologous to mitochondrial genes, including COX1 (Corral et al., 1989; Farrelly & Butow, 1983; Jacobs & Grimes, 1986). However, no studies have demonstrated any functional significance to the nuclear copies of these duplicated mitochondrial genes. In our twofold coverage of shotgun sequence for the serotype A genome of *C. neoformans* we found partial sequences of 360 and 80 bp of COX1 in nuclear DNA but there was no evidence for a complete gene. It is likely that this partial duplication in the nuclear genome has no functional significance.

The capture of the COX1 transcript by differential display RT-PCR occurred probably because of the extensive AU-rich composition of mitochondrial mRNAs (Pon & Gottfried, 1991) and despite the fact that *C. neoformans* mitochondrial genes like COX1 lack poly(A) tails (Groot et al., 1974). A Northern analysis confirmed the upregulation of COX1 at 37 °C. In fact, two transcripts hybridized to the COX1 probe, implicating a processed (1–9 kb) and a pre-processed (2–3 kb) form. However, only the larger 2–3 kb transcript was notably overexpressed at 37 °C. The existence of both pre-processed and processed forms of mitochondrial mRNAs, including the COX1 transcript, has been described previously in several fungi (Agsteribbe & Hartog, 1987; Burger et al., 1985; Costanzo & Fox, 1990; Dyson et al., 1989; Grivell, 1989; Ozinga et al., 1984; Tracy & Stern, 1995), plants (Barkan, 1988; Gray & Lovello, 1993; Tracy & Stern, 1995; Wolff & Kuck, 1996) and animals (Ojala et al., 1980; Tracy & Stern, 1995). These pre-processed or precursor RNAs comprise polycistronic transcripts that have not yet been processed into smaller, intermediate and mature forms of RNA. The dicistronic and tricistronic transcripts, which carry mRNAs encoding COX1, ATP9 and ATP8, could also serve as immediate templates for protein synthesis and thereby augment the expression of these proteins, especially during high temperature growth or other stress conditions. In fact, translation of dicistronic templates has been reported in several other systems (Barkan, 1988; Gualberto et al., 1988; Ohmen et al., 1990).

This common occurrence of transcriptional units in mitochondrial DNA from a variety of plants (Barkan, 1988; Tracy & Stern, 1995; Wolff & Kuck, 1996) and fungi (Agsteribbe & Hartog, 1987; Burger et al., 1985; Costanzo & Fox, 1990; Dyson et al., 1989; Grivell, 1989; Ozinga et al., 1984; Tracy & Stern, 1995) implicates their role in facilitating the co-ordinate regulation of gene expression. In our study, the overexpression of a single transcriptional unit encoding ATP9, COX1 and ATP8 mRNAs in...
C. neoformans suggests that at elevated host temperatures the serotype A strain (H99) upregulates expression of this transcriptional unit. However, an alternative mechanism for the increased numbers of transcripts may be related to a reduction in the rate of mRNA processing. The pathological significance of host-temperature-induced overexpression or accumulation of the COXI pre-processed 2-3 kb mRNA is unclear but this transcriptional unit is detected in vivo. The COXI expression profile is most likely related to a stress response for this pathogen and emphasizes the potential importance of oxidative phosphorylation and energy production as the yeast fights for survival in the host. In fact, it has been well documented in mammalian cells (Bond, 1988; Kay et al., 1987) and other eukaryotes, including Drosophila (Yost et al., 1990; Yost & Lindquist, 1988), Dictyostelium (Maniak & Nellen, 1988) and Saccharomyces (Yost & Lindquist, 1991), that cellular stresses, such as the heat-shock response, block mRNA processing resulting in the accumulation of precursor mRNAs. Furthermore, the increased numbers of COXI transcripts in the serotype A strain (H99) at high temperature growth follows a similar pattern of another gene in a C. neoformans oxidative pathway. An alternative oxidase pathway has been shown to be functional in C. neoformans and our recent studies show that the AOX1 (alternative oxidase) gene is similarly increased in its transcriptional expression at 37°C compared to 30°C growth in serotype A (H99) yeast cells (McDade et al., 2002). Taken together, these observations of elevated transcription of oxidative genes (COXI and AOX1) suggest that they are part of the stress response by C. neoformans induced by a high temperature environment like the host. Similarly, Steen et al. (2002) during a global screen of C. neoformans with SAGE observed an increased gene expression with an elevated environmental temperature of mitochondrial genes such as ubiquinol–cytochrome-c reductase and those associated with oxidation such as the antioxidant superoxide dismutase.

The combined findings that temperature (37°C) had no affect on COXI transcription in a serotype D C. neoformans strain (JEC21) and that a serotype A strain (H99) had no introns in COXI to splice were surprising. First, we demonstrated that the presence or absence of introns in COXI is not absolutely serotype-specific. For example, introns 1 and 2 of COXI were present in one serotype A strain, whereas two serotype D strains lacked intron 3 of COXI. However, these results do implicate that some genetic drift of COXI actually occurred after the evolutionary emergence of these two separate varieties (serotypes A and D). Serotype D strains carry more introns than serotype A strains, which appear to be losing them.

Furthermore, there were also differences in processing and stability of the mature COXI transcript between serotype A (H99) and D (JEC21) strains. We did not detect evidence for monocistronic transcripts for COXI in H99, although mature single COXI transcripts were detected in JEC21. Since monocistronic transcripts encoding the flanking ATP9 and ATP8 genes were detected in H99, this finding suggests that monocistronic COXI transcripts are likely to be present in H99, but are highly unstable. These differences in temperature-related COXI expression between the two serotypes are consistent with a recent study which showed with SAGE that a serotype A strain had 12% of its tags significantly different when grown between 25 and 37°C, but in a serotype D strain there was only a 4-9% difference. In this expression profile study, specific gene expression patterns between serotypes A and D were not concordant (Steen et al., 2002). The differences between serotypes A and D with respect to the COXI structure and regulation emphasize that despite similarities in producing clinical disease and their ability to produce genetic hybrids, these serotypes genetically diverged from each other approximately 18 million years ago (Xu et al., 2000b).

It was of interest to us whether these differences in mitochondrial structure and transcription between JEC21 and H99 might translate into a difference in phenotype. For instance, serotype D yeast cells of JEC21 have a slower rate of growth at elevated temperatures (37°C) when compared to H99 and in fact died at 39°C. This finding concurs with a recent study in which Martinez et al. (2001) examined 38 strains of serotypes A and D and found that, although there was some overlap, serotype D strains were generally more susceptible to thermal killing than serotype A strains. Furthermore, serotype D yeast cells of 3501, the parent strain of JEC21, are avirulent in the rabbit model of cryptococcal meningitis (Perfect et al., 1980) with host temperatures approaching 39–40°C.

We hypothesize that the mitochondrial differences in gene structure, regulation and high temperature growth might translate into pathobiological significance between serotypes A and D. For instance, there has been some correlation between intronic characteristics and pathogenesis for the entomopathogenic fungus Beauveria brongnartii (Neveuglise et al., 1997). Furthermore, in heat-shock genes there has been an evolutionary move towards intron-less genes. It has been suggested that during high temperature exposure or growth there would be inefficiencies in splicing and hence the absence of introns would reduce the amount of translated aberrant polypeptides (Yost et al., 1990). There could be an advantage for an organism to possess genes without introns or have translational competence of unspliced, polycistronic messages at high temperature. Thus, we approached the function of serotype D mitochondria with multiple introns at higher temperatures by producing AD hybrids with the selected ability to grow well at high temperatures (37–39°C). In creating these cryptococcal hybrid strains, we took advantage of the known mitochondrial inheritance from the MATa strain (Xu et al., 2000a) to create hybrid strains that grew at 37°C and contained only serotype D mitochondria. The growth of these strains with serotype D mitochondria at high temperature was the same as the parental serotype A strain containing serotype A mitochondria. These results suggest that intron-splicing
of the COXI transcript occurs normally during high temperature growth. Neither the upregulation of the COXI transcript nor the need for its splicing appear to influence high temperature growth, and thus the differential regulation of COXI between serotypes may not have an impact on the pathogenesis of Cryptococcus neoformans. Congeneric pairs of serotype A MATa and MATa strains are being created. When these are available, we can make AD hybrids with serotype A mitochondria and formally test the impact of serotype A versus D mitochondria on Cryptococcus neoformans pathogenesis.

In summary, these studies have shown that the mitochondrial gene COXI in Cryptococcus neoformans var. grubii (H99, serotype A) is encoded on a polycistronic message, regulated by environmental temperature, expressed in host tissue and has no introns. It is likely induced as part of the stress response for this strain. In contrast, Cryptococcus neoformans var. neoformans (JEC21, serotype D) COXI is not regulated by temperature and contains multiple introns. However, an AD hybrid strain containing only the serotype D mitochondria was able to grow similarly to the parental serotype A strain with serotype A mitochondria and formally test the impact of serotype A versus D mitochondria on Cryptococcus neoformans pathogenesis.

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D. L. Toffaletti and others


Expression of COX1 in C. neoformans


